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Animal Model

MALToma-Like Lesions in the Murine Gastric Mucosa after Long-Term Infection with *Helicobacter felis*

A Mouse Model of Helicobacter pylori*-Induced Gastric Lymphoma*

Angelina Enno,* Jani L. O'Rourke,† C. Rolfe Howlett,* Andrew Jack,‡ Michael F. Dixon,‡ and Adrian Lee†

From the School of Pathology and School of Microbiology and Immunology,† University of New South Wales, Sydney, Australia; and Academic Unit of Pathology,‡ University of Leeds, United Kingdom*

The long-term consequences of helicobacter infection were observed in an established murine model of human helicobacter infection. Stomachs of specific pathogen-free BALB/c mice infected with* Helicobacter felis *were examined for inflammation with particular reference to lymphoid cell proliferation and lymphoepithelial lesions. There was little evidence of an inflammatory response in animals sacrificed up to 19 months after infection. In contrast, from 22 months, 38% of infected animals had lymphoid follicles, whereas no lymphoid follicles were found in noninfected control animals. Lymphoepithelial lesions were observed in 25% of infected mice compared with none in controls. Immunostaining confirmed the B-cell nature of the lymphoid infiltrate. The morphology of these lesions closely resemble those seen in human gastric MALToma. This animal model would provide an opportunity to study the pathogenesis of lymphoproliferative disease. (Am J Pathol 1995, 147:217–222)

The normal stomach is virtually devoid of lymphoid tissue.[1] However, a lymphoid infiltrate with features of mucosa-associated lymphoid tissue (MALT) is characteristic of chronic *Helicobacter pylori* gastritis. In particular, the presence of hyperplastic lymphoid follicles has been attributed to a specific B-cell response to *H. pylori*.[2,3] In a study by Genta et al,[4] lymphoid follicles were found exclusively in *H. pylori*-infected patients with none observed in non-infected subjects.

Before the discovery of *H. pylori*, Isaacson[5] noted that low-grade B-cell gastric lymphomas had histological appearances similar to that of lymphomas arising at other sites of acquired MALT such as the thyroid and salivary gland. Indeed, these lesions were collectively referred to as MALTomas. Given the realization that *H. pylori* infection was the cause of the acquired MALT in the stomach, it is not surprising that a link between *H. pylori* gastritis and low-grade B-cell gastric lymphoma has been sought.

Epidemiological evidence comes from a prospective serological study that showed that individuals with *H. pylori* infection had a sixfold increased risk for the subsequent development of gastric lymphoma while there was no significant difference in prior *H. pylori* infection between individuals with extra-gastric non-Hodgkin's lymphoma and controls.[6] More compelling evidence supporting the role of the bacterium in the pathogenesis of gastric lymphoma has been

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Address reprint requests to Professor Adrian Lee, School of Microbiology and Immunology, The University of New South Wales, Sydney, Australia 2052.

provided by the demonstration that eradication of *H. pylori* with antimicrobial therapy resulted in regression of lymphomas.[7]

In 1987, we isolated the bacterium *H. felis* from cat stomach.[8] This close relative of *H. pylori* was shown to colonize the mouse stomach, and the *H. felis*-infected mouse has been developed as a small animal model of human helicobacter infection.[9] In a long-term study of *H. felis* infection in specific pathogen free (SPF) BALB/c mice, we found lesions in the stomach that strongly resemble human *H. pylori*-associated gastric B-cell lymphoma. The morphological aspects of these lesions are the subject of this report.

Materials and Methods

Experimental Animals

Female 7- to 8-week-old SPF BALB/c mice were obtained from the Animal Breeding and Holding Unit of The University of New South Wales, Australia, and maintained on a diet of sterile commercial food pellets (Clarel Holdings, Northbridge, NSW). Sterile water was given *ad libitum*.

Bacteria

H. felis (ATCC 49179 strain CS1) was grown on Blood Agar Base No. 2 (Oxoid, Basingstoke, UK) with lysed horse blood (Oxoid, West Heidlberg, Australia), 5% v/v, and containing amphotericin B (Fungizone, Squibb, Princeton, NJ), 2.5 mg/L; trimethoprim (Sigma, St Louis, MO), 5 mg/L; polymyxin (Sigma), 1.250 IU/L; and vancomycin (Eli Lilly, West Ryde, Australia) 10 mg/L. Plates were incubated in a microaerophilic atmosphere at 37°C for 48 hours.

Infection with H. felis

260 mice were infected with *H. felis* introduced into the stomach p.o. via plastic tubing three times over a 5-day period with $\sim 10^9$ organisms/ml.

Histological Examination

Groups of 20 infected mice were killed at 2 to 3 monthly intervals for up to 26 months after infection. Groups of 10 uninfected mice from the same original batch were killed at each time point. Longitudinal strips of the stomach and proximal duodenum (usually three or four strips) were fixed in 10% buffered formalin. In the 22 to 26-month groups the spleen and slices of liver were also taken for histology. Paraffin-embedded sections were cut, stained with H&E, coded and examined "blind" by MFD and AE. The presence of lymphoid aggregates (circumscribed collections of lymphocytes) and follicles (aggregates containing germinal centers) was recorded and the sites noted. Two forms of lymphoepithelial (LE) lesions were distinguished, one comprising glandular infiltration by individual atypical lymphocytes which was designated "early," and established LE lesions where the epithelium contained large clusters of atypical lymphocytes or lesser degrees of infiltration associated with epithelial destruction and reactive changes.

Immunohistology

Samples of stomach from selected mice were frozen in liquid nitrogen; 4 μm frozen sections were cut and acetone fixed. Immunofluorescence was used to detect macrophages using the monoclonal antibody MI/70.15.1 (Serotec, Oxford, UK) at a dilution of 1:10, CD3 (Serotec clone KT3; dilution 1:5) and T-cell subsets CD4 (Serotec clone KT9; dilution 1:5) and CD8 (Serotec clone KT15; dilution 1:10). Immunoperoxidase staining with the avidin-biotin complex method (Vectastain Elite kit, Vector Laboratories, Loughborough, UK) was used with a pan-B cell marker (Serotec clone LR6.2B6D6.C9; dilution 1:200), surface immunoglobulin staining with biotinylated anti-mouse immunoglobulin (Vector Laboratories; dilution 1:200), and rat anti-mouse transferrin receptor (Serotec clone ER-MP 21; dilution 1:200) for activated/cycling cells. We did not stain for immunoglobulin light chains, as >95% of immunoglobulin expressed in normal mouse lymphoid tissue contains κ chains and it is therefore difficult to interpret light chain restriction by immunohistochemistry.[10] Immunoperoxidase-stained frozen sections were developed with diaminobenzidine (Aldrich, Castle Hill, Australia) and counterstained with Harris' hematoxylin.

Results

Up to 19 months there was little inflammation in the gastric mucosa of any of the animals, infected or control. *H. felis* organisms were most numerous in the antral mucosa with lesser numbers in the gastric epithelium at the junction with the squamous portion of the stomach and very few in the corpus mucosa. Small numbers of lymphocytes and polymorphs were found in both control and infected mice, and there was no significant difference in the grades of chronic

inflammation, activity and surface epithelial degeneration between the two groups. The earliest change in terms of lymphoid proliferation was seen at 22 months when small aggregates of mature lymphocytes were found in the lamina propria, particularly in the narrow zone of junctional mucosa close to the squamous portion of the stomach and extending into adjacent corpus.

From 22 months a marked difference in lymphoid elements was observed between infected and non-infected animals (Table 1). The stomachs of some infected mice had marked nodularity (Figure 1a) as a consequence of large lymphoid aggregates in the mucosa and submucosa (Figure 1b). The larger lymphoid follicles contained poorly defined germinal centers composed of centroblasts with scattered tingible body macrophages and indistinct mantle zones. While lymphoid aggregates were found in a small proportion (21%) of control mice compared with 64% of test animals, lymphoid follicles were seen only in infected animals (38%). In the overlying mucosa, there was scanty lymphocyte, eosinophil, and polymorph infiltration of the epithelium while numerous plasma cells and macrophages were evident in the superficial lamina propria. Small numbers of eosinophils were identified within the diffuse lymphoid infiltrates.

The surface or foveolar epithelium overlying lymphoid aggregates was hyperplastic with occasional mild nuclear atypia. With increasing size the lymphoid aggregates were associated with glandular loss in the proximal corpus. In some animals "invaginations" of the surface/foveolar epithelium into the submucosa resulted in complex branching structures surrounded by a dense monomorphic lymphocytic infiltrate. The invaginated epithelium was invariably hyperplastic and sometimes sufficiently atypical to be termed dysplastic. Epithelial dysplasia was always related to the invaginations of mucosa surrounded by proliferated lymphoid tissue and was not seen in control mice. Clusters of organisms were frequently seen in the lumina of these deep invaginations. In some instances, very large lymphoid aggregates with large, ill-defined germinal centers extended beyond the submucosa into the main muscle coat. Occasional lymphoid aggregates, usually in the subserosa, developed a nodal structure with peripheral sinusoids and bordering histiocytes, but other subserosal nodules did not possess definite germinal centers. There was intimate association between lymphoid cells and epithelium with moderately large centrocyte-like cells in close contact with foveolar epithelium.

As the cellular infiltrate became more monomorphic there was loss of the normal T and B cell zones discernible in normal mucosa-associated lymphoid tissue. Infiltration of the surface foveolar epithelium by the centrocyte-like cells produced early LE lesions (Figure 1c). In parallel with the development of frank LE lesions showing destruction of epithelium (Figure 1d), the lymphoid infiltrate became more monomorphic with loss of germinal centers, but an increase in tingible body macrophages. In some cases the infiltrate was composed of blast cells, while others show a range of cytological appearances from centrocyte-like to monocytoid cells.

Lymphoepithelial lesions were found in 25% of the infected animals compared with none in the controls. They were found mainly in the corpus mucosa immediately distal to the squamo-columnar junction, an area heavily colonized by *H. felis* in the older animals (Figure 1e). As the area of involvement enlarged, so greater amounts of corpus mucosa were replaced by sheets of lymphoid cells containing scattered LE le-

Table 1. *Histopathology of the Gastric Mucosa of Long-Term H-felis-infected BALB/c Mice*

Time post-infection (*months*)	Number of mice	Follicles (%)		Lymphoid aggregates (%)	Early LE lesions (%)	Established LE lesions (%)
		Antrum	Body			
Uninfected						
0 to 19	95	0	0	0	0	0
22	10	0	0	0	0	0
23	10	0	0	10	0	0
24	10	0	0	20	0	0
26	18	0	0	39	0	0
Infected						
0 to 19	167	0	0	0	0	0
22	20	5	50	50	5	30
23	19	0	47	37	0	16
24	10	0	0	70	10	20
26	31	23	10	87	6	16
Total 22 to 26 months	80	10	28	64	5	25

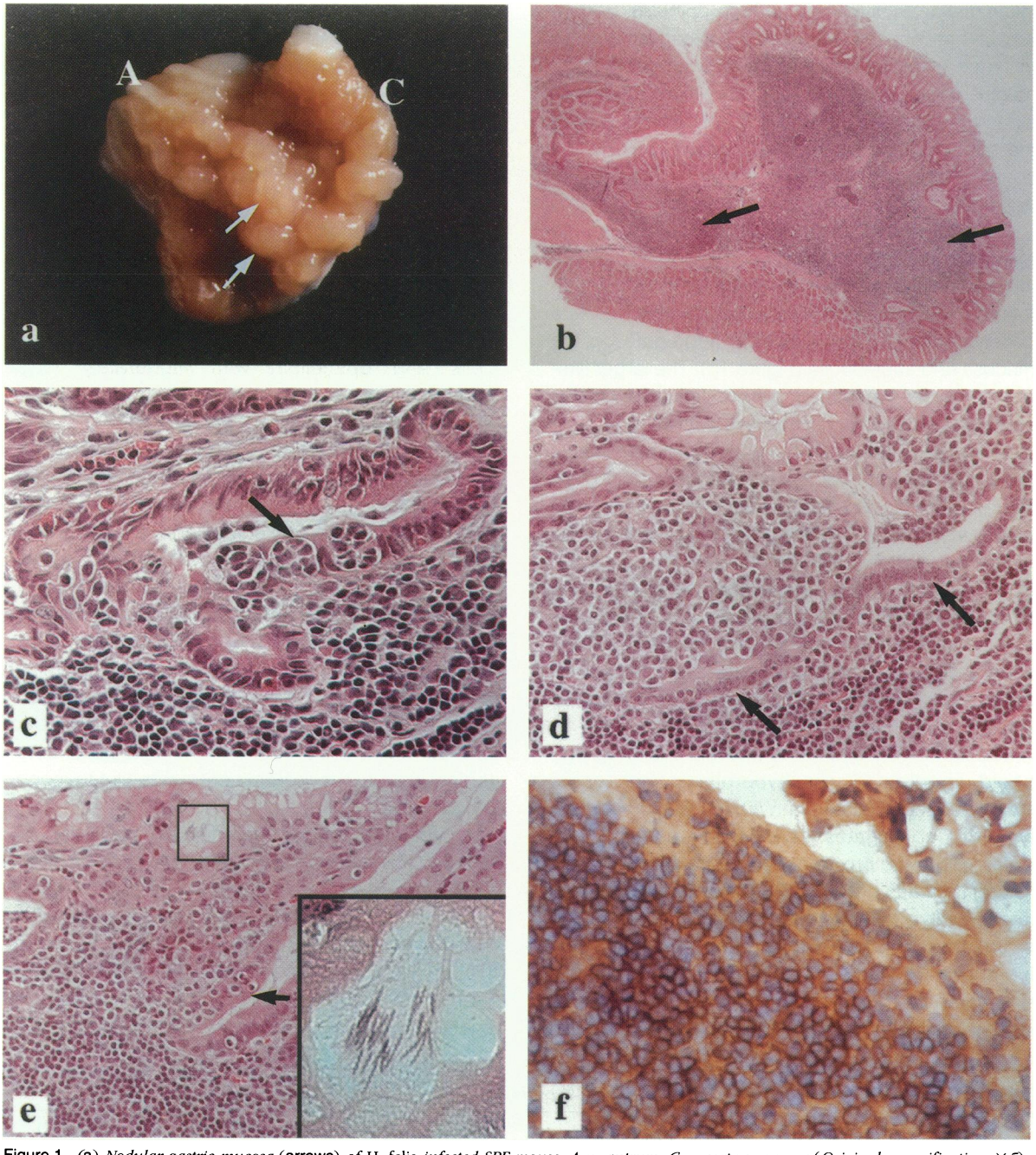

Figure 1. (a) *Nodular gastric mucosa* (arrows) *of* H. felis*-infected SPF mouse; A = antrum; C = corpus mucosa* (*Original magnification,* ×5). (b) *Section through nodular mucosa showing lymphoid hyperplasia in the submucosa. Poorly demarcated germinal centers* (arrows) *are apparent within the lymphoid tissue* (*H&E; original magnification,* ×13). (c) *Small aggregates of centrocyte-like cells* (arrows) *infiltrate hyperplastic foveolar epithelium in this relatively early lymphoepithelial* (*LE*) *lesion* (*H&E; original magnification,* ×100). (d) *Fully developed LE lesion showing destruction of glandular and foveolar epithelium with a central epithelial remnant* (arrows)(*H&E; original magnification,* ×63). (e) *Destructive and early LE lesions* (arrow) (*H&E; original magnification,* ×63) *with* (inset) *long, spiral shaped* H. felis *organisms* (*H&E; original magnification,* ×250). (f) *Immunoperoxidase-stained frozen section of late LE lesion showing surface immunoglobulin expression by the majority of lymphocytes* (*ABC; original magnification,* ×130).

sions. Occasional foci of lymphoid infiltration and LE lesions were found in antral mucosa in a small number of animals, again associated with dense colonization by *H. felis*. Some of the most advanced tumors were seen at 22 months rather than in the last group of mice to be sacrificed at 26 months. We attribute this to bias in sampling, as the least healthy mice were selected at 22 months.

Immunohistochemistry

Immunohistochemical staining for surface immunoglobulin and for the pan-B-cell antigen confirmed the predominantly B-cell nature of the lymphoid proliferation (Figure 1f). The infiltrate also contained small numbers of T-lymphocytes that were mainly of the CD4 subset. Macrophages were scattered in the lamina propria and were concentrated around the periphery of the lymphoid aggregates.

Discussion

In this study we have shown that *H. felis* infection in BALB/c mice can produce lesions that strongly resemble human gastric MALToma (low-grade B-cell lymphoma of the stomach). The lesions produced in mice were similar to the human lesions in several important respects including the morphology of the B-cell infiltrate, localization in the stomach, and the presence of lymphoepithelial lesions identical to those seen in human MALTomas. There was evidence that the MALToma-like lesions in the mouse were evolving from hyperplastic gastric lymphoid tissue, which is a further parallel with the human disease. It is rare to find a pathogenic agent that is able to reproduce the histological features of a human tumor so closely in another species, and this would suggest that *H. felis* infection in BALB/c mice may be an important model for the study of cellular and molecular events in the pathogenesis of MALToma.

In addition to the MALToma-like lesions, the squamo-columnar junctional zone was also the site of invaginated gastric foveolar epithelium. Infolding of the gastric epithelium was always associated with lymphoid proliferation. This phenomenon could be a response to mucosal damage by *H. felis*, since they were often found in the glands lining the flask-like structure. However, elevation of the mucosa by lymphoid nodules could also render the tissue susceptible to mechanical stress, and the invaginations could represent herniations into the submucosa. Nevertheless, the morphology of the epithelial elements suggest a more fundamental lymphoid-epithelial interaction. The epithelium was invariably hyperplastic and on occasions dysplastic. Indeed in one case the complex branching architecture combined with cytological atypia gave an appearance indistinguishable from well differentiated adenocarcinoma. Coexistent lymphoma and adenocarcinoma in the human stomach is rare but has been the subject of occasional case reports.[11–13]

In human stomachs *H. pylori* causes a combination of lymphoid hyperplasia with cell injury and acute on chronic inflammation. In BALB/c mice, *H. felis* caused lymphoid hyperplasia with almost no evidence of acute gastritis. In these mice helicobacter infection appears to be acting as a source of chronic antigenic stimulation. The lack of acute inflammation after helicobacter infection appears to be a strain-specific effect in mice. Other strains such as Swiss/Quackenbush mice infected with *H. felis* show severe gastric mucosal injury and inflammation resulting in atrophic gastritis without development of MALToma.[14] The reasons for these inherited differences in host response to helicobacter are not clear. An important question that may be answered by this model is whether a particular balance between active chronic gastritis and lymphoid hyperplasia represents the optimal conditions for the development of MALToma.

Studies of gastric MALToma have raised a number of interesting questions about the definition of lymphoma. A general feature of neoplasms is that they do not resolve when the causative agent is removed. The observation that MALTomas may resolve after helicobacter eradication therapy would appear to argue against MALTomas being true neoplasms; a similar phenomenon is seen in some cases of immunoproliferative small intestinal disease.[15] However, a more appropriate way to formulate this question is whether the development of MALTomas requires only chronic immune stimulation by helicobacter in a susceptible host or whether genetic change is required to make the transition from lymphoid hyperplasia to lymphoma. Recent work in human MALTomas, which has shown that allele imbalance at a number of tumor suppressor gene loci occurs during this transition from gastritis to MALToma, would support the idea that genetic change is required.[16]

Monoclonality is generally regarded as a feature of lymphomas. Attempts have been made to investigate immunoglobulin heavy chain gene arrangements in formalin-fixed paraffin-embedded tissue but because of the poor sensitivity of the PCR technique used this was not successful. In the future we intend to investigate the evolution of clonality in the developing lymphoid lesions in frozen tissue from prospective studies.

The abrupt marked appearance of lymphoepithelial lesions at 22 months is unexplainable but may at least be attributed to the fact that this group of animals included those that were noticeably ill. Before this, lymphoepithelial lesions were observed in one mouse at 16 months. Nevertheless, histopathology indicated

an evolving lesion, and perhaps the long latent time is a reflection of the neoplastic proliferation.

Spontaneous lymphomas are known to appear in aged mice;[17] in particular the average incidence is after 20 months in BALB/c mice. In this study equal numbers (16%) of animals in test and control groups developed lymphoma in the spleen, in contrast to gastric MALTomas which were observed only in infected animals, with none observed in control animals. The histopathology of splenic lymphomas were all similar and, in terms of human classifications, would be regarded as diffuse large cell lymphomas of B-cell type. The cell morphology of these lymphomas was easily distinguished from the lesions seen in the stomach. The exact molecular basis for the presence of splenic lymphomas in aged mice is not known, but similar mechanisms could act in the helicobacter-stimulated gastric lymphocytes to cause MALTomas. This may explain the sharp onset after 22 months infection. Another possible cause of genetic damage in human MALToma is the production of reactive oxygen intermediates in the course of cell injury and acute inflammation.[18,19] Given the data presented above this would seem to be an unlikely explanation in the BALB/c mouse. Further investigation of these mechanisms could give important insights into how the cellular context in which a genetic abnormality occurs can influence the phenotype of the resulting tumor. A question of particular importance is whether further genetic change can lead to autonomy from helicobacter stimulation.

Although caution is always required in using mouse models for human tumors the preliminary data presented here would suggest that important questions about the interaction of immune responses and genetic change could be answered using the *H. felis*-infected mouse model.

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